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ORIGINAL ARTICLE

Is there a pathological relationship between microalbuminuria and *Helicobacter pylori* vacA and cagA genes in type 2 diabetic patients?

Latifeh Abdollahi · Zahra Molabashi · Mehdi Azami · Rasoul Salehi · Seyed Hossein Hejazi · Narghes Abdali

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Abstract Diabetes is one of the most common metabolic disorders worldwide. Microalbuminuria, one of the complications following elevated glucose levels, is used as a simple and effective method of kidney function analysis in diabetic patients. Early stage diagnosis of microalbuminuria is useful in monitoring and prevention of the progression of diabetic nephropathy. Due to a lower immunity, diabetic patients are more susceptible. Reports have shown that cytotoxin-associated gene A (cagA)-positive *Helicobacter pylori* is one of the effective factors responsible in microalbuminuria pathology. However, a lack of information on the relationship between microalbuminuria in type II diabetes and vacuolating cytotoxin A (vacA) is evident. The present study aimed at the relationship between microalbuminuria in type II diabetes and vacA and cagA genes from *H. pylori*. A total of 88 type II diabetic patients referred to the Isfahan Endocrine and Metabolism Research Center participated in this study. Nested PCR was performed to exclude host genes, and consequently, *H. pylori* genotyping was performed based on vacA and cagA. Out of the 88 patients, 68.2 % (60/88) of them have microalbuminuria. A total of 18.2 % of the patients were infected with *H. pylori* in which 75 % of them showed microalbuminuria and 18.8 % of this group had simultaneously microalbuminuria and expression of cagA. The association between microalbuminuria and *H. pylori* infection was not statistically significant (p=0.52). Considering the population size and criteria of choice, statistical analysis did not show any significant relationship between the virulence genes vacA and cagA of *H. pylori* and microalbuminuria in type II diabetic patients.

Keywords Type II diabetics · Microalbuminuria · *H. pylori* · vacA gene · cagA gene

Introduction

Diabetes mellitus is a metabolic disease characterized by elevated levels of blood sugar and a disorder in the metabolism of carbohydrates, fats, and proteins (Association 2013). The prevalence and its complications have turn diabetes to one of the important diseases with the most significant health, social, and economical problems worldwide. According to the World Health Organization reports, the expected population of diabetics will increase from 171 million in 2000 to 366 million in 2030 (Whiting et al. 2011). Also, studies show that the prevalence of diabetes will increase 7/7 % in 2025 (Shaw et al. 2010). In Iran, about 4 million people have diabetes, and on average, 500,000 are diagnosed with diabetes annually (Federation 2006). In 2001, studies estimated 1.6 million diabetic patients in the population over 20 years of age in Iran. Experts have predicted 5,125,000 incidences of type II diabetes in 2025 (Abdollahi et al. 2012). During the past two

L. Abdollahi · M. Azami · S. H. Hejazi
Skin Diseases and Leishmaniasis Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

L. Abdollahi · Z. Molabashi
Department of Microbiology, Qom Branch, Islamic Azad University, Qom, Iran

R. Salehi (✉)
Department of Anatomy and Molecular Biology, Pediatric Diseases Research Center, School of Medicine, Isfahan University of Medical Sciences, Isfahan 81745, Iran
e-mail: r_salehi@med.mui.ac.ir

S. H. Hejazi
Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

N. Abdali
Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran

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decades, an increased end-stage renal disease (ESRD) has been observed among patients with diabetes, especially type II. Previous studies have reported the incidence of nephropathy up to 47 % among patients with type II diabetes (Papazafiropoulou et al. 2010).

Few studies have been conducted to elucidate the influence of specific genotypes of *Helicobacter pylori* in the development of microalbuminuria and due to the fact that patients with diabetes are at a lower level of immunity and therefore at risk for *H. pylori* infection. *H. pylori* may have a role in the prevalence of microalbuminuria in these patients (Bener et al. 2007; Ko et al. 2001). It is speculated that pathogenic strains of *H. pylori* antigens cause an immune response in endothelial cells, resulting in a pathological process leading to the excretion of albumin (Ibrahim et al. 2010). One of the major virulence factors, present in about 50 % of *H. pylori* strains, is the vacA cassette causing vacuoles and ultimately death in mammalian cells (Farshad et al. 2008; Supajatura et al. 2002). The vacA gene consists of two subunits: s (signal) and m (middle), and each is composed of two parts, 1 and 2. The s1/m1 is the characteristic type creating the highest inflammatory response (Sasaki et al. 2009). Discovered in 1989, the cytotoxin-associated gene A (cagA) cassette is another virulence factor of *H. pylori* (Blaser and Atherton 2004). This is unique to *H. pylori* and has no other similar gene among the rest of bacteria (Van Doorn et al. 1998; Sicinschi et al. 2003). cagA encodes genes that are of type IV secretion system. cagA is able to induce an increase in inflammatory cytokines such as interleukin-1, -6, and -8; tumor necrosis factor alpha (TNF-α); and vascular permeability growth factor (VPGF) (Blaser and Atherton 2004; Godoy et al. 2003), which are associated with the excretion of albumin and microalbuminuria. In fact, these inflammatory cytokines increase the vascular permeability of the glomerular membrane, thus leading to the loss of albumin (Schalkwijk and Stehouwer 2005; Ibrahim et al. 2010).

Thus, given that a large percentage of the population are diabetics, as well as these individuals are at a higher risk for *H. pylori* infection, and given the importance of early detection and prevention of microalbuminuria in diabetic nephropathy, in this study, we evaluated the relationship between virulence genes vacA and cagA of *H. pylori* in patients with type II diabetes with microalbuminuria.

Material and methods

This study was confirmed by the Isfahan University ethical committee, and a signed informed consent was filled by each participant. In this cross-sectional study, stool sample was collected from 88 type II diabetic patients referring to Isfahan Endocrine and Metabolism Research Center, Isfahan, Iran. We checked high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglyceride (TG) levels for all of the study samples with at least 8-h fasting period. Also, blood pressure (systolic and diastolic) of each patient was recorded. The patients were divided into groups: type II diabetes with microalbuminuria and without microalbuminuria. Microalbuminuria was defined as two positive urine samples with an albumin-to-creatinine ratio of 30:300 in the past 3 months. Stool samples were collected in containers and stored in −70 °C until used for DNA extraction. DNA was extracted from stool samples using a DNA extraction kit (QIAamp Stool Mini Kit) according to the manufacturer's instructions and was stored at −20 °C. DNA samples were analyzed using specific 16 s ribosomal RNA (rRNA) primers (Table 1) to ensure the absence of PCR inhibitors.

Amplification reactions were performed in 25-μl volume containing 2.5-μl 10× PCR buffer, 200 mM deoxyribonucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, 100 ng DNA, and 10 pM of each primer. Amplification condition is shown in Table 1. The following nested PCR was then performed for the detection of *H. pylori* in 25-μl volume containing 5 μl of 10× PCR buffer, 200 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 U of Taq polymerase, 100 ng DNA, 10-pM external (first round) and internal primers with 3 μl of the PCR product from the first stage used. cagA and vacA genes were examined according to the protocol as described previously (Falsafi et al. 2009). The list of primers and amplification condition for each gene and PCR step is shown in Table 1. Finally, the PCR products were analyzed using 1.5 % agarose gel containing 0.5 mg/ml of ehidium bromide in Tris-borate-EDTA buffer at 90 V for 1 h. The products were examined under ultraviolet illumination.

Results

A total of 88 patients with type II diabetes participated in this study in which 68.2 % (60/88) and 31.8 % (28/88) of these presented with and without microalbuminuria, respectively.

Using DNA extracted from stool samples and PCR amplification of the 16 s rRNA gene, the amount of obtained DNA was calculated to be 92 % (Fig. 1). The process was repeated for samples with no amplification; otherwise, the samples were replaced or excluded. The prevalence of *H. pylori* in stool specimens was 18.2 % (16/88) using ureC primers (Fig. 2 and Table 2). Seventy-five percent (12/16) of *H. pylori*-infected patients showed microalbuminuria, and 18.8 % (3/16) had simultaneous microalbuminuria and expression of cagA gene (Table 2).

The results showed that although microalbuminuria is higher in patients with *H. pylori* infection, the association between microalbuminuria and *H. pylori* infection was not statistically significant (p=0.52) (Table 2). In addition, the incidence of microalbuminuria was higher in cagA-positive

Table 1 Oligonucleotide primers and PCR condition used for amplification of different genes

PCR condition	Product size (bp)	Primer sequences	Target gene
16 s rRNA	5′-CCTACGGGAGGCAGCAGTAG-3′	500	94 °C 5 min (1 cycle)
	5′-CAACAGAGCTTTACGATCCGAAA-3′		94 °C 30 s, 60 °C 30 s, and 72 °C 30 s for 35 cycles
			72 °C 1 min (1 cycle)
H. pylori ureC gene	First round	250	First round: 94 °C 5 min (1 cycle), 94 °C 45 s, 56 °C 45 s, and 72 °C 45 s for 15 cycles
	5′-AAGCCTTTAGGGGTGTTAGGGGTTT-3′ 5′-AAGCCTACTTTCTAACACTAACGC-3′		72 °C 5 min (1 cycle)
	Second round	250	Second round: 94 °C 5 min (1 cycle), 94 °C 30 s, 64 °C 30 s, and 72 °C 30 s for 45 cycles
	5′-CTTTCTTCTCAAGCAATTGTC-3′ 5′-CAAGCCATCGCCGGTTTTAGC-3′		72 °C, 5 min (1 cycle)
cagA	5′-AATACACCAACGCCTCCA-3′	400	94 °C 4 min (1 cycle)
	5′-TTGTTGCCGCTTTTGCTCTC-3′		94 °C 1 min, 59 °C 1 min, and 72 °C 1 min for 35 cycles
			72 °C 10 min (1 cycle)
vacA (s)	5′-ATGGAAATACAACAAACACAC-3′	S1=259	94 °C 4 min (1 cycle)
	5′-CTGCTTGAATGCGCCAAAC-3′	S2=286	94 °C 1 min, 52 °C 1 min, and 72 °C 1 min for 35 cycles
			72 °C 10 min (1 cycle)
vacA (m)	5′-CAATCTGTCCAATCAAGCGAG-3′	M1=570	94 °C 1 min, 52 °C 1 min, and 72 °C 1 min for 35 cycles
	5′-GCGTCTAAATAATTCCAAGG-3′	M2=642	72 °C 10 min (1 cycle)

s signal, *m* middle

samples; however, a statistically significant relationship was not found between microalbuminuria and cagA gene expression ($p=0.08$) (Table 2 and Fig. 3). We could found vacA gene in one patient (Fig. 4 and Table 2). No significant relationship was observed between vacA gene and microalbuminuria ($p>0.65$). The results showed the higher prevalence of microalbuminuria in young patients (30–40 years of age) ($p=0.02$). Figures 1, 2, 3, and 4 showed PCR products of different genes that amplified in this study.

Independent *t* test revealed no significant relationship between microalbuminuria and serum HDL, LDL, and TG and blood pressure (Table 2). There is statistically significant relationship between sex and microalbuminuria ($p=0.03$). It is noteworthy that women (22.2 %) are more susceptible to infection with *H. pylori* than men (11.85) ($p=0.04$) (Table 2).

Discussion

Diabetic nephropathy is one of the most important kidney dysfunction factors in diabetes mellitus and also one of the most common causes of kidney failure. In fact, it is responsible for 30 % of cases of kidney failure. The disease is identified via proteinuria, high blood pressure, and kidney failure (Rashki et al. 2009; Talaei et al. 2011). Different stages of diabetic nephropathy include increased glomerular filtration,

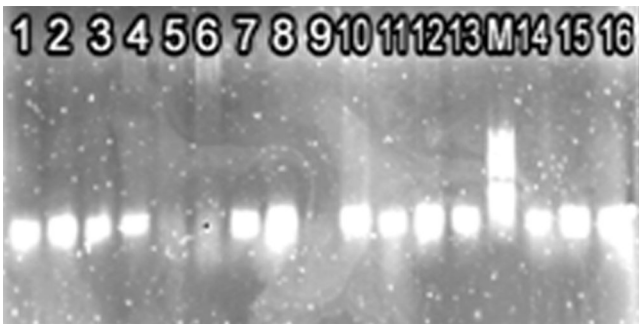

Fig. 1 PCR product of the 16 s rRNA gene using specific primers on the 1/5 % agarose gel. *Lanes 5*, *6*, and *9* show that DNA extracted from stool samples could not amplify. *Lane M* molecular weight markers (50 bp)

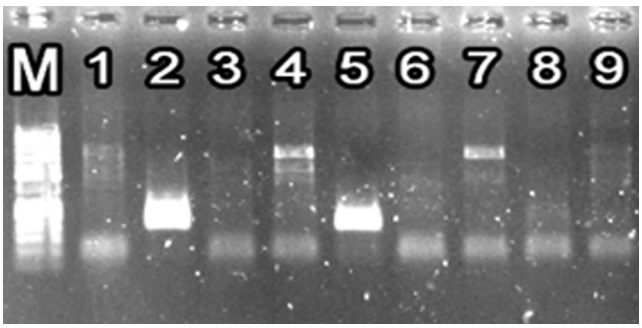

Fig. 2 PCR product of the ureC gene using specific primers on the 1/5 % agarose gel. *Lanes 2* and *5* show samples infected with *H. pylori*. *Lane M* molecular weight markers (50 bp)

Table 2 Variables that were checked in patients with and without microalbuminuria

Variables	Microalbuminuria		*p* value
	Positive (%)	Negative (%)	
H. pylori infection			
Positive	12 (75)	4 (25)	0.52
Negative	48 (66.7)	24 (33.3)	
cagA gene expression			
Positive	3 (100)	0 (0)	0.08
Negative	9 (69.2)	4 (30.8)	
Gender			
Male	20 (58.8)	14 (41.2)	0.03
Female	40 (74.1)	14 (25.9)	
Age (year)	48.8±7.5	54±9.8	0.02
HDL (mg/ml)	41.9±9.02	41.8±11.6	0.99
LDL (mg/dl)	95.4±32.6	105.2±35.2	0.12
Triglyceride	136.8±52.9	159.8±88.7	0.11
Systolic pressure (mmHg)	11.81±1.5	11.8±1.36	0.98
Diastolic pressure (mmHg)	7.4±0.86	7.2±0.95	0.53

HDL high-density lipoprotein, *LDL* low-density lipoprotein

microalbuminuria, macroproteinuria, and kidney failure (Talaei et al. 2011). Long before the onset of clinical nephropathy, microalbuminuria occurs which is an early stage of diabetic kidney problems and a predictor of future kidney failure. The main treatment of diabetic nephropathy is prevention, and microalbuminuria is the easiest most sensitive indicator. Early stage diagnosis of microalbuminuria is a helpful indicator in diabetic disease management (Afkhami ardekani et al. 2004; Rossi et al. 2008). The reported prevalence of microalbuminuria in type II diabetic patients in Isfahan, Ahvaz, and Tehran has been 16.6, 35.2, and 20.3 %, respectively (Ariabod et al. 2009; Alamdari et al. 2006).

Multiple immune disorders, especially humoral and cellular dysfunction in diabetic patients, lead to further chronic

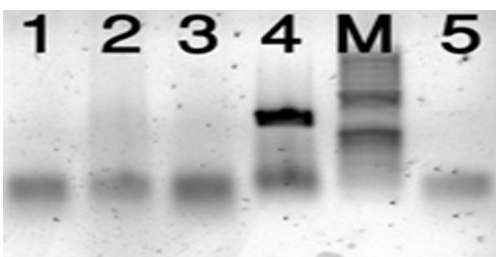

Fig. 3 PCR product of the cagA gene using specific primers on the 1/5 % agarose gel. *Lane 4* shows cagA gene in the DNA samples. *Lane M* molecular weight markers (50 bp)

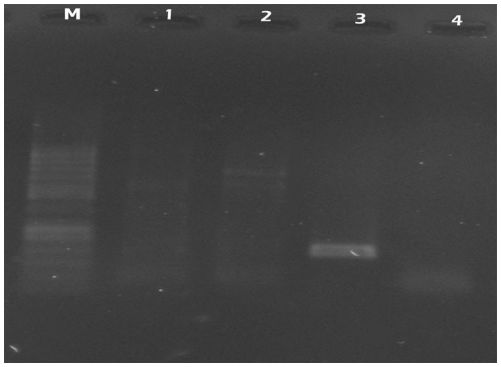

Fig. 4 PCR product of the vacA gene using specific primers on the 1/5 % agarose gel. *Lane 4* shows vacA gene in the DNA samples. *Lane M* molecular weight markers (50 bp)

infections such as *H. pylori*. Also, reduced gastrointestinal motility in diabetic patients can increase bacterial colonization and growth (Ibrahim et al. 2010). *H. pylori* is the most common bacteria that have afflicted the human societies worldwide. *H. pylori* has a frequency of 90 % in Japan (Salih 2009), and 82–92 % occurrence has been reported in Iran (Supajatura et al. 2002). In a study in Kurdistan province of Iran, the incidence of *H. pylori* was about 36.5 %, which is lower than other regions of the world (Yazdanpanah et al. 2009). *H. pylori* incidences have been estimated in other regions of Iran; for example in the northwest city of Ardabil, the incidence was found to be 47.5 % and that in the central part of Iran Yazd and Semnan showed 30.6 and 48 %, respectively (Mikaily et al. 1999; Moradi and Rashidy-Pour 2000; Alamdari et al. 2006). *H. pylori* is a population dynamic infection, in which more crowded areas have higher prevalence of infection (Moradi and Rashidy-Pour 2000; Mokhtari 2002). Also, the incidences increase with age, though these could be due to the individuals who carried the bacteria since infancy and thus are seropositive.

Using the same method, in two independent studies in France and Germany on stool samples, the occurrences of *H. pylori* were 25.4 and 39.2 %, respectively (Sasaki et al. 2009). In other studies, the prevalence of *H. pylori* positive stool samples was 80 % for developing and 30 % for developed countries (Mitchell et al. 2003). There are ambiguous results on incidences of *H. pylori* in diabetic and healthy individuals.

In a study conducted in Turkey, diabetic had a significantly higher rate of infection with *H. pylori* than nondiabetic individuals. An interesting finding in this study was the relationship between *H. pylori* infection with diabetic neuropathy, although the difference was not significant (Demir et al. 2008). In a population study in Italy, a significant relationship was observed between *H. pylori* infection in diabetic and nondiabetic women; however, the difference was not observed in men (Quadri et al. 2000). The results confirm the influence of gender in the incidences of *H. pylori* infection. We also

found a higher prevalence of *H. pylori* infection in women. In a recent study in Turkey, the prevalence of microalbuminuria in type II diabetic patients with *H. pylori* infection was significantly higher compared with noninfected patients (Tanriverdi 2011). Considering that inflammatory markers in patients with *H. pylori* infection were also significantly higher, they have concluded that *H. pylori* infection may cause a systemic inflammatory response; therefore, it is considered to be a risk factor for the progression of diabetic nephropathy (Tanriverdi 2011).

In this study, genotyping of *H. pylori* in diabetic patients with microalbuminuria done revealed that cagA-positive genotype is more likely to be associated with the development of microalbuminuria. It is possible that a larger number of samples to obtain will show a more accurate estimate in our patients. This could be the fact that certain genotypes of bacterium are responsible for microalbuminuria. The significant relationship of *H. pylori* cagA-positive genotypes with microalbuminuria has also been reported in other studies (Pietroiusti et al. 2006; Ibrahim et al. 2010).

The reported prevalence of *H. pylori* infection in our study was 18.2 %, which seems that the population criteria and the choice of sample can be effective. A recent study showed that *H. pylori* infections in children 1 to 7 years, in the densely and sparsely populated areas in Isfahan, Iran, have been about 64 and 31 %, respectively (Mokhtari 2002). Furthermore, the incidence of *H. pylori* infection in French patients has been reported as 25.4 % (Sasaki et al. 2009).

It seems that the parameters of the study population only focused on patients referred to Isfahan Endocrine Research Center and were not randomly selected from the community, therefore may have affected the prevalence obtained in this study. It is possible that our patients were selected from inhabitants of sparsely populated areas, or such as the study done in France, obtained stool samples may have affected the outcome. Simultaneous presence of the cagA and vacuolization alleles of vacA seems to increase the pathogenicity of *H. pylori*. Epidemiological and animal model studies have revealed that most cagA- and vacA-positive strains have the potential toxicity and virulence inducing the secretion of interleukin (IL)-1 in epithelial cells (Farshad et al. 2008). In fact, the cag pathogenicity island (cag-PAI) cassette contains 29 genes coding type IV secretory structures which are able to transfer 120-kDa CagA protein into the gastric epithelial cells. After entrance, CagA is phosphorylated and tabbed to HSP-2 tyrosine phosphate which causes a cellular response similar to growth factor and cytokine production by the host. In infected individuals, levels of IL-1β, IL-2, IL-6, IL-8, and TNF-α are elevated (Suerbaum and Michetti 2002). IL-8 has a pivotal role in the inflammatory process; however, it has been shown that a greater response to *H. pylori* is related to cag-PAI. This response is associated with the activation of NF-κB and AP-1 (Suerbaum and Michetti 2002). Chronic induction of the inflammatory response is an important risk factor in the development of arteriosclerosis and kidney problems. Therefore, it is speculated that *H. pylori* cagA-positive infection is responsible for the extensive endothelial damage seen in patients with infection and microalbuminuria (Pietroiusti et al. 2006). The formed cagA antibody can react with endothelial antigens and consequently vascular lesions and leakage of albumin. VacA also induces the production of TNF-α, IL-1β, αIL-1, IL-6, IL-10, and IL-13 (Sasaki et al. 2009). The role of inflammatory cytokines, such as IL-1, -6, and -8 in pyelonephritis, has been confirmed. Studies have shown that inhibition of B cell leukemia/lymphoma-2 (BCL-2) gene in mice leads to abnormalities such as polycystic kidney. Thus, the relationship between BCL-2 gene expression and renal disease should be considered (Nakayama et al. 1994). On the other hand, other studies have shown that *H. pylori* infection leads to decreased expression of BCL-2 gene in the target cells (Mojtahedi et al. 2007). *H. pylori* $cagA^+/vacA^+$ show a greater reducing effect than $cagA^-/vacA^+$ strains. Therefore, a correlation exists between vacA-positive genotype, decreased expression of BCL-2, and *H. pylori* infection and kidney problems. Also, kidney failure is associated with some ILs (Mojtahedi et al. 2007; Nakayama et al. 1994).

In conclusion, further studies with large patient's series and involving many inflammatory markers and antigenic molecules may put forth a discussion on another absolute indication of *H. pylori* eradication. With a larger sample size, the effects of vacA gene on microalbuminuria in type II diabetic patients infected with *H. pylori* may show more convincing results. *H. pylori* has a higher incidence in patients with type 2 diabetes who have microalbuminuria.

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Conflicting interest None declared.

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